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13. ABSTRACT *(Maximum 200 Words)*

The hypothesis of this research is that specific cell-binding proteins or peptides can be used to enhance (therapeutic) DNA delivery to breast carcinomas; this hypothesis is being experimentally tested.

During the period covered by this progress report, we have continued our analyses of the novel αvβ3-binding proteins which we first described in our previous annual report. These were developed by the directed mutagenesis of a natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), and subsequent screening of a library of these molecules, usingphage display technology. A novel derivative of FNfn10 was identified and shown to bind with high affinity and specificity to purified αvβ3 integrin. It also interacted with cell surface-expressed αvβ3, as determined by flow cytometry, but did not bind detectably to other cell surface integrins.

Overall, these experiments have provided important tools and insights that will enhance gene transfer to breast carcinomas.

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FOREWORD

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INTRODUCTION

A number of gene delivery systems, including virally-based vectors as well as non-viral methods, are presently being explored as potential DNA delivery vehicles for gene- and immuno-therapy of breast cancer. However, currently available DNA delivery vehicles for gene therapy of breast cancer have a very wide host cell range, making it difficult to specifically target them to tumor cells. Therefore, the experiments that were performed under the auspices of this grant award were aimed at developing innovative new approaches that will allow one to selectively target DNA molecules to breast cancer cells. The underlying hypothesis which we explored was as follows: that one can use specific protein or peptide sequences to selectively target linked DNA molecules to breast cancer cells. This hypothesis was explored by a combination of approaches, including the use of peptide phage display libraries and recombinant adenovirus-derived gene delivery systems.

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Analysis of DNA delivery by adenovirus penton base proteins (AdPB) (months 1 - 12)
- *Task 2.* Application of phage display technology to the identification of breast cancer targeting peptides (months 1 - 15). *Timeline extended to 1-36 months.*
- *Task 3.* Analysis of DNA delivery to breast cancer cells by novel peptides (months 16-30). *Timeline extended to 16-36 months.*
- *Task 4.* Studies of DNA delivery using an *in vivo* xenograft model for breast cancer (months 25-36). *Originally included in the approved tasks, but ultimately deferred until a future date, when more compelling in vitro data exist. The adenovirus penton base proteins developed in Aim 1 did not show sufficient promise to merit performance of these in vivo studies. This decision was made for two major reasons. First, the AdPB proteins mediated DNA delivery with a lower level of efficiency than commercially available reagents such as lipofectamine. Second, DNA delivery by AdPB required the formation of unstable trimolecular complexes, incorporating AdPB, DNA and a bridging peptide. It was deemed highly unlikely that a tirmolecular complex of this kind, held together by low-affinity peptide-DNA and peptide-protein interactions would remain intact in vivo We therefore decided to extend Task 2, because we obtained exciting preliminary data in these experiments. In particular, we focussed our attention on the derivation of more promising lead molecules, with the ability to interact with key cell surface receptors expressed on breast cancers (i.e., $\alpha v\beta 3$ integrin and CD40; see below).*

Research Accomplishments associated with the above tasks

Task 1: Experiments on the analysis of DNA delivery by adenovirus penton base proteins were reported in a previous annual report. We have not performed any work with these materials during the period covered by the present report.

Task 2: Experiments aimed at the identification of novel breast cancer targeting peptides generated results that included the following, previously unreported, findings:

- We conducted directed mutagenesis of a natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), and subsequent screening of a library of these molecules, using phage display technology. A novel derivative of FNfn10 was identified and shown to bind with high affinity and specificity to purified $\alpha v\beta 3$ integrin. It also interacted with cell surface-expressed $\alpha v\beta 3$, as determined by flow cytometry, but did not bind detectably to other cell surface integrins. These results are summarized below and in greater detail in the manuscript by Richards and coworkers (see Appendix; *Richards J., et al. A fibronectin type III domain modified with a novel RGDWXE consensus sequence binds with enhanced affinity and specificity to human $\alpha v\beta 3$ integrin. Submitted for publication*).

Background: Why Target αvβ3?

Alphavbeta3 (CD51/CD61) is a member of the integrin family of cell surface adhesion receptors. Over 20 different αβ integrin heterodimers exist, each with different tissue and ligand specifities. Normal tissue distribution of αvβ3 is generally limited to high levels of expression on osteoclasts, with lower levels observed on platelets, megakaryocytes, kidney, vascular smooth muscle, placenta, dendritic cells, and in varying amounts on normal endothelium.

αvβ3 integrin is a multifunctional cell surface receptor that has pleiotropic roles in normal cell growth and survival, and which can contribute to oncogenesis. Consistent with this, upregulation of αvβ3 expression has been observed on the endothelial cells of angiogenic vessels, and binding of αvβ3 to the basement membrane is a critical step in the angiogenesis induced by basic fibroblast growth factor and tumor necrosis factor alpha (5). Expression of αvβ3 has also been implicated in tumor invasion, and it has been shown that αvβ3 binds matrix metalloproteinase-2 (MMP-2) and presents MMP-2 on the surface of invasive carcinomas and on invasive angiogenic endothelial cells (2, 7).

αvβ3 also regulates cell growth and survival, since ligation of this receptor can, under some circumstances, induce apoptosis in tumor cells (6). Furthermore, disruption of cell adhesion with anti-αvβ3 antibodies, RGD peptides, and other integrin antagonists has been shown to slow tumor growth (1, 3, 4). Finally, the selective upregulation of αvβ3 expression on tumor blood vessels is also being explored as the basis for imaging of neoplastic lesiosn, and the αvβ3-specific antibody LM609 has been successfully used for this purpose *in vivo* (8).

Novel molecules capable of binding with high specificity to αvβ3 integrin have potential utility in several applications, and as a consequence, αvβ3 is an important target for drug discovery and selection of new binding ligands. The tenth fibronectin type III domain (FNfn10) was developed as a phage display scaffold because of its small size (94 residues), monomeric assembly, and ability to retain its folded β-sheet morphology while exposed loops were randomized. In addition, FNfn10 lacks cysteine residues and requires no post-translational modification, allowing for large-scale bacterial expression. We reasoned that since FNfn10 binds αvβ3 *in vivo*, affinity maturation of the RGD sequence in the exposed FG loop might result in a modified FNfn10 with high-affinity for αvβ3. We further reasoned that a modified derivative of FNfn10 might be particulatly useful for vector or gene delivery applications because of (1) the small size of this protein, (2) its simple monomeric structure, and (3) its lack of disulfide bonds. These considerations suggested to us that it ought to be possible to place a modified FNfn10 on the surface of bacteriophage or virus vectors (for DNA delivery applications) and also to derivatize the protein in such a way as to link it directly to plasmid DNA molecules. As such, the modified FNfn10 protein might be expected to have major advantages over the adenovirus penton base protein (AdPB), which emerged as a somewhat more difficult reagent, due in part to its larger size, disulfide bonds and propensity to form multimers.

Summary of findings

As noted above, we have utilized a natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), as a scaffold for the selection of novel αvβ3-binding molecules. By randomizing residues surrounding the RGD sequence in the flexible FG loop of FNfn10, we selected αvβ3-binding modified FNfn10 clones with a novel RGDWXE consensus sequence. One of these clones, 3JCLI4-FNfn10, binds with high affinity and specificity to purified αvβ3 integrin. It also interacts with cell surface-expressed αvβ3, as determined by flow cytometry, but does not bind detectably to other cell surface integrins. Taken together, these data show that 3JCL14-FNfn10 is

a specific, high-affinity αvβ3-binding protein which may have utility in future applications involving the targeting of αvβ3-positive cells.

The complete results from this work are included in the Appendix, in the manuscript by Richards et al.

KEY RESEARCH ACCOMPLISHMENTS OF DURING THIS PERIOD

- Utilization of a natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), as a scaffold for the selection of novel αvβ3-binding molecules and successfiul identification of novel integrin-binding proteins using this method. Briefly, by randomizing residues surrounding the RGD sequence in the flexible FG loop of FNfn10, we selected αvβ3-binding modified FNfn10 clones with a novel RGDWXE consensus sequence. One of these clones, 3JCLI4-FNfn10, binds with high affinity and specificity to purified αvβ3 integrin. It also interacts with cell surface-expressed αvβ3, as determined by flow cytometry, but does not bind detectably to other cell surface integrins. Taken together, these data show that 3JCL14-FNfn10 is a specific, high-affinity αvβ3-binding protein. These properties, combined with the small, monomeric, cysteine-free and highly stable structure of 3JCLI4-FNfn10, may make this protein useful in future applications involving the targeting of αvβ3-positive cells.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: See Bibliography

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training was provided for Ms. Michelle Miller was provided (Ms. Miller is a 1999 college graduate, who has been working on this project as a laboratory technician). It is expected that Ms. Miller will attend graduate school within the next 1-2 years, to obtain her Ph.D. (personal communication from Ms. Miller), and the present research experiences should assist her in that goal. Research training for two summer undergraduate researchers, Ms. Johanna Abend and Ms. Laura McLane, was also provided. Ms. Abend s long-term goals include graduate (Ph.D.) education — a goal which will be enhanced by her training under this award. Ms. McLane s long-term goals are similar, although she is presently employed as a laboratory technician at Emory University.

CONCLUSIONS

The conclusions which can be drawn from this period of research are as follows:

- A natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), can be successfully used as a scaffold for the selection of novel αvβ3-binding molecules. By randomizing residues surrounding the RGD sequence in the flexible FG loop of FNfn10, we selected αvβ3-binding modified FNfn10 clones with a novel RGDWXE consensus sequence. One of these clones, 3JCLI4-FNfn10, was shown to bind with high affinity and specificity to purified αvβ3 integrin. It also interacted with cell surface-expressed αvβ3, as determined by flow cytometry, but did not bind detectably to other cell surface integrins. Thus, we were able to derive a novel, specific, high-affinity αvβ3-binding protein. This protein is small, monomeric, cysteine-free and highly stable, which may make it very useful in future applications involving the targeting or detection of αvβ3-positive cells (such as those found in breast tumor vasculature).

So What Section

The knowledge gained from these experiments advances the basic goals proposed in the original grant application, and provides important tools and insights into approaches which can be used to selectively deliver therapeutic DNA to breast cancers.

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BIBLIOGRAPHY (PUBLICATIONS)

These materials are all also included as Appendices

Manuscripts:

J. Richards, M. Miller, A. Koide, S. Koide, S. Dewhurst. A fibronectin type III domain modified with a novel RGDWXE consensus sequence binds with enhanced affinity and specificity to human αvβ3 integrin. Submitted for publication in the Journal of Biological Chemistry, 2002.

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LIST OF PERSONNEL

List of personnel who were supported from the research effort during this period

Individual	**Role**
Stephen Dewhurst, PhD	PI
John Frelinger, Ph.D.	CoPI
Michelle Miller, B.S.	Technician
Johanna Abend, B.S.	Summer undergraduate researcher

APPENDIX MATERIALS

Award Number:	DAMD17-99-1-9361
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PRINCIPAL INVESTIGATOR:	Stephen Dewhurst, Ph.D.
CONTRACTING ORGANIZATION:	University of Rochester Medical Center Rochester, New York 14642
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To improve the safety and efficacy of viral vectors in vaccine and gene therapy studies, it is advantageous to exercise control over the target cell tropism of the vectors. Since phage display technology allows for the selection of receptor-specific ligands, this technique has been adapted to screening for ligands to alpha$_v$beta$_3$ integrin. Our goal is to find both high-affinity binding ligands as well as ligands which will trigger receptor internalization. The cell adhesion receptor alpha$_v$beta$_3$ ($\alpha_v\beta_3$) integrin is a target of interest because of its expression on endothelial, dendritic, and cancer cells; its role in angiogenesis; and its involvement in the entry of many viruses (including adenovirus) into host cells. Previous work using phage display methods has resulted in the identification of short $\alpha_v\beta_3$-binding peptides containing the RGD consensus motif. However, we believe that it may be possible to select for higher affinity interactions using a small protein scaffold. Fibronectin is a natural ligand for $\alpha_v\beta_3$, which is compromised of multiple domains. The tenth fibronectin type III domain (FN3) of human fibronectin is a very stable, small (< 100 amino acids), structurally defined molecule with an immunoglobulin-like structure and a loop containing the RGD sequence. We have established phage display systems for FN3 [Koide et al. (1998) J. Mol. Biol. 284:1141]. By randomizing the residues in the FG loop of FN3 in a library displayed on M13 bacteriophage, sequences binding with much higher affinity to $\alpha_v\beta_3$ were selected, all of which contain a consensus sequence. This FN3 library has also been screened for internalization into selected cell lines of interest. Future applications may include not only gene therapy/vector development but also tumor inhibition and tumor detection/visualization.

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A fibronectin type III domain modified with a novel RGDWXE consensus sequence binds with enhanced affinity and specificity to human αvβ3 integrin

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Running Title: Fibronectin type III domain with enhanced binding to αvβ3

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Fibronectin type III domain with enhanced binding to αvβ3

Summary

Alphavbeta3 integrin is a cell surface receptor involved in normal cell growth, attachment, and migration; it has also been implicated in tumor invasion and angiogenesis and is of considerable interest as a target for therapeutics and gene delivery. In the present work, we have utilized a natural integrin-binding protein, the tenth fibronectin type III domain (FNfn10), as a phage display scaffold for the selection of novel αvβ3-binding molecules. By randomizing residues surrounding the RGD sequence in the flexible FG loop of FNfn10, we selected αvβ3-binding modified FNfn10 clones with a novel RGDWXE consensus sequence. One of these clones, 3JCLI4-FNfn10, binds with high affinity and specificity to purified αvβ3 integrin. It also interacts with cell surface-expressed αvβ3, as determined by flow cytometry, but does not bind detectably to other cell surface integrins. In contrast to the disintegrin echistatin, which inhibits both αvβ3- and αIIbβ3-mediated cell adhesion, 3JCLI4-FNfn10 inhibits only αvβ3-specific attachment. Taken together, these data show that 3JCL14-FNfn10 is a specific, high-affinity αvβ3-binding protein that can inhibit αvβ3-dependent cellular processes. These properties, combined with the small, monomeric, cysteine-free and highly stable structure of 3JCLI4-FNfn10, may make this protein useful in future applications involving detection and targeting of αvβ3-positive cells.

Fibronectin type III domain with enhanced binding to αvβ3

Introduction

Alphavbeta3 (CD51/CD61) is a member of the integrin family of cell surface adhesion receptors. Over 20 different αβ integrin heterodimers exist, each with different tissue and ligand specificities. Normal tissue distribution of αvβ3 is generally limited to high levels of expression on osteoclasts, but is also observed on platelets, megakaryocytes, kidney, vascular smooth muscle, placenta, dendritic cells(1), and in varying amounts on normal endothelium (reviewed in (2)). In contrast to α5β1, which binds to only fibronectin, αvβ3 binds to a wide range of RGD-containing integrin ligands, including but not limited to fibronectin, vitronectin, osteopontin, von Willebrandt factor, and fibrinogen(2).

αvβ3 integrin is a multifunctional cell surface receptor that has pleiotropic roles in normal cell growth and survival, and which can contribute to oncogenesis. Consistent with this, upregulation of αvβ3 expression has been observed on the endothelial cells of angiogenic vessels, and binding of αvβ3 to the basement membrane is a critical step in the angiogenesis induced by basic fibroblast growth factor and tumor necrosis factor alpha(3). Expression of αvβ3 has also been implicated in tumor invasion, and it has been shown that αvβ3 binds matrix metalloproteinase-2 (MMP-2) and presents MMP-2 on the surface of invasive carcinomas and on invasive angiogenic endothelial cells(4,5).

αvβ3 also regulates cell growth and survival, since ligation of this receptor can, under some circumstances, induce apoptosis in tumor cells(6). Furthermore, disruption of cell adhesion with anti-αvβ3 antibodies, RGD peptides, and other integrin antagonists has been shown to slow tumor growth(7-9). Finally, the selective upregulation of αvβ3 expression on tumor blood vessels is also being explored as the basis for imaging of neoplastic lesiosn, and the αvβ3-specific antibody LM609 has been successfully used for this purpose *in vivo*(10)

Fibronectin type III domain with enhanced binding to αvβ3

Novel molecules capable of binding with high specificity to αvβ3 integrin have potential utility in several applications, and as a consequence, αvβ3 has been a frequent target for drug discovery and selection of new binding ligands. Phage display technology, in which combinatorial peptide libraries are expressed on the surface of bacteriophage, has been used to select for peptide ligands capable of binding to αvβ3 -- yielding a wide range of RGD-containing peptide sequences capable of interacting at moderate affinity with αvβ3 and other integrins(11,12). Phage-displayed random peptide libraries have also been constructed and screened using framework proteins such as the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). This resulted in the identification of phage clones which could be used to stain human umbilical vein endothelial cells in a flow cytometric assay. However, the ability of the purified recombinant CTLA-4 protein to stain cells and its cross-reactivity with other integrins was not reported(13).

Fibronectin is a natural ligand of integrins, and it consists of repeats of three types of domains. The tenth fibronectin type III domain (FNfn10) contains the RGD sequence in the highly flexible loop connecting the F and G β-strands (FG loop) (14,15). FNfn10 was developed as a scaffold for phage display of peptides because of its small size (94 residues), monomeric assembly, and ability to retain its global fold while exposed loops were randomized(16). In addition, FNfn10 lacks cysteine residues and requires no post-translational modification, allowing for large-scale bacterial expression. We reasoned that since FNfn10 binds αvβ3 *in vivo* via the RGD sequence, modification of amino acid residues surrounding the RGD sequence in the FG loop might result in a modified FNfn10 with higher affinity and/or specificity for αvβ3. In this study, we demonstrate that derivatives of FNfn10 with high affinity and specificity for αvβ3 can be selected from a partially randomized FNfn10 M13 phage library, expressed on the surface of phage M13. All αvβ3-binding FNfn10 clones were found to contain a RGDWXE

consensus sequence, and the purified monomeric FNfn10-derived proteins were found to have similar characteristics to αvβ3-specific monoclonal antibodies, with respect to their stability, specificity, affinity, and effects on cell adhesion.

Experimental Procedures

General reagents--- All chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO). Purified integrins, cell adhesion strips, and LM609 were purchased from Chemicon (Temecula, California). Echistatin and GRGDSPK peptide were obtained from Bachem (Torrence, California).

Cell Culture--- K562, K562-αvβ3, K562-αIIbβ3, K562-αvβ5, K562-α4β1, and K562-α4β7 cells were a gift of Dr. S. Blystone (Upstate Medical University, Syracuse, NY)(17). K562 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma), 0.5U/L penicillin-streptomycin, and 2mM L-Glutamine (Gibco). K562-αvβ3, K562-αIIbβ3, K562-αvβ5, K562-α4β1, and K562-α4β7 cells were maintained in the same media containing 500 μg/ml G418 (GENETICIN) (Gibco).

Construction of phage vectors---We constructed a phage-display vector for FNfn10, JCFN, by cloning the FNfn10 gene(16) in the modified M13 vector, JC-M13-88(18). The FNfn10 gene was amplified from pAS38 (16) using oligonucleotides FN3JCM8Nhe (CCTAGCTAGCGTAGCTCAGGCCATGCAGGTTTCTGATGTTC) and FN3JCM8Hin (GGCCAAGCTTGACCGCCACCAGAACCGCCACCGGTACGGTAG), digested with the restriction enzymes NheI and HindIII, and subcloned in JC-M13-88 using the same restriction enzyme sites so that the FNfn10 gene is placed in-frame between the OmpA signal sequence and M13 geneVIII. We confirmed that the JCFN phages prepared using *E. coli* XL-1blue displayed FNfn10 on the phage surface by ELISA (data not shown). Purified FNfn10 clones were expressed with an N-terminal histag and a C-terminal GKKGK tag. The GKKGK tag was incorporated to increase the solubility (16) and to serve as a preferred biotinylation site. FNfn10 genes were amplified using oligonucleotides FN1F2 (CGGGATCCCATATGCAGGTTTCTGATGTTCCGCGTGACCTGGAAGTTGTTGCTGCGACC) and FN3GKKGK (CCGACTCGAGTTACTATTTACCTTTTTTACCGGTACGGTAGTTAATCGAG), digested

with the restriction enzymes NdeI and XhoI, and ligated with pAS45 (16) digested with the same restriction enzymes. The expression vector for the wild type protein, pAS54, contained an Arg6 to Thr mutation that had been introduced to remove a secondary Thrombin site (16).

Construction of the FNfn10 library---We constructed a FNfn10 library, JCFN-RGD, in such a way that the FG loop has XRGDXXXX sequence where X stands for any amino acids (residue #77-84; residue numbering is according to Figure 2(a) of Koide et al. (1998) (16)). Mutagenesis on the JCFN template was performed according to Kunkel's method(19) using an oligonucleotide JCFNFGRGD (GTTAATCGAGATTGGCTTGGAMNNMNNMNNMNNATCGCCGCGMNNAGTAACAGCGTATAC, where N is a mixture of A, G, C and T, and M is a mixture of A and C). *E. coli* SS320 was electro-transformed with the mutagenesis mixture, resulting in a phage library containing 1.5×10^9 independent clones. The phages were re-amplified in *E. coli* XL1-blue with various concentrations of isopropyl β-D-thiogalactopyranoside (IPTG).

αvβ3 biopanning using the JCFN-RGD library--- Integrin biopanning was performed similarly to the method previously described(11). Purified αvβ3 (5μg/ml in TBS buffer containing 2 mM $CaCl_2$) was bound to a single microtiter well of a 96-well plate. The well was rinsed 3 times with sterile water containing 2 mM $CaCl_2$ and blocked with 5% phage blocking reagent (Novagen, Darmstadt, Germany) for one hour at room temperature. The plate was rinsed 5 times with TBS buffer containing 0.1% Tween-20 and 2 mM $CaCl_2$, then incubated with JCFN-RGD library diluted in 5% blocking reagent for one hour. The plate was washed ten times with TBS buffer containing 0.1% Tween 20 and 2 mM $CaCl_2$ and bound phage were eluted with 0.2 M glycine-HCl (pH 2.2) containing 1 mg/ml BSA for 10 minutes at room temperature with gentle agitation and neutralized with 15% (v/v) of 1 M Tris-HCl (pH 9.1). The phage were amplified using the *E. coli* ER2738 host strain (New England Biolabs, Beverly, MA) in the presence of 1mM, 0.1 mM or with no IPTG and purified by precipitating with 20 % (w/v) polyethylene glycol-8000, 2.5 M NaCl and resuspending in PBS containing 20 % (v/v) glycerol. The biopanning and amplification of

eluted phage were repeated for two more rounds. Upon completion of three rounds of biopanning, individual phage clones were amplified and the genomes were purified by phenol extraction. Polymerase chain reaction was performed with the primers SFN3F (AGCTCAATTGGTCCGGTGGAGGTTCTGATGTTCCGCGTGACCTG) and SFN3R (AGCTAAGCTTTTAGGTACGGTAGTTAATCGAGAT) to amplify the FNfn10 domain. The DNA was sequenced using the SFN3F primer.

Protein preparation---The expression of FNfn10 proteins was performed as previously described (16) except that the proteins were further biotinylated. After biotinyation, the protein-containing solution was applied to a nickel affinity column and unbound materials were washed off; the column was then equilibrated with 50mM sodium phosphate buffer (pH8.0) containing 500mM sodium chloride. D-biotinoyl-ε-aminocaptoic acid N-hydroxysuccinimide ester (726μM) (Boehringer Mannheim, Mannheim, Germany) in the same buffer was applied to the column, and the column was incubated for an hour at room temperature to perform biotinylation. This biotinylation process was performed a total of three times. Then the column was washed with 20mM Tris-HCl buffer (pH8.0) containing 500mM sodium chloride, and the biotinylated FNfn10 was eluted with the buffer containing 500mM imidazole. The proteins purified in this manner were >90% pure as judged by SDS-PAGE and reverse phase chromatography. Protein concentrations for subsequent analyses were determined by Bradford assay and confirmed by SDS-PAGE analysis and Coomassie Brilliant Blue staining. Biotinylation was confirmed by Western analysis with streptavidin-horseradish peroxidase (Oncogene, Cambridge, MA).

ELISA detection of biotinylated FNfn10 clones--- Purified integrin was bound to a flat-bottom, high-binding EIA/RIA plate (Corning Costar, Corning, NY) at a concentration of 5 μg/ml in TBS for 12-16 hours at 4 °C. The plate was washed once with TBS and blocked for 2 hours at room temperature with 0.1M $NaHCO_3$, 0.5 mg/ml BSA, 0.2% NaN_3, pH 8.6. Biotinylated FNfn10 clones were bound in TBST+ Ca^{++} (TBS 0.1% Tween-20, 2mM $CaCl_2$). Plates were washed ten times with TBST+ Ca^{++} and incubated 20 minutes with 2 μg/ml streptavidin-horseradish peroxidase (Oncogene) in TBST+ Ca^{++} at room temperature. Plates were

washed ten times with TBST+ Ca^{++}. Bound peroxidase was detected with ABTS (2,2'-azino-di-3-ethyl-benzthiazoline sulfonic acid) peroxidase substrate (Sigma), 1 mg/ml in 0.1 M NaCitrate, 0.1 M Na_2HPO4, pH 4.0 supplemented with 0.03% hydrogen peroxide. Color was allowed to develop for 10-15 minutes and the A_{400} was read with a SpectraCount ELISA reader (Packard, Downers Grove, IL).

Flow cytometric detection of cell-binding by biotinylated FNfn10 and FNfn10-3JCLI4--- K562 cells (10^5) transfected with various integrins were resuspended in 5 ml FACS buffer (PBS, 0.5% BSA, 0.1% NaN_3), washed, and resuspended in FACS buffer containing either 0.4 μg/ml FNfn10 protein (wild-type of 3JCLI4) or 1 μg/ml LM609 , prior to incubation at room temperature for 20 minutes. Cells were then washed with 5 ml FACS buffer, incubated in 1:50 streptavidin-APC (BD Pharmingen, San Diego, California) for 20 minutes and washed again with 5 ml FACS buffer, prior to detection of cell surface fluorescence using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California).

Cell adhesion assays--- Fibronectin and vitronectin-coated strips (Chemicon) were rehydrated 15 minutes in PBS. Inhibitor in IMDM/10% FBS was added to the bottom of the well, and 10^5 cells/well in maintenance media were added to a final volume of 100 μl/ well. Cells were incubated 2 hrs at 37°C, 5% CO_2, washed three times with PBS at room temperature, and stained 10 minutes with 0.2% crystal violet, 10% ethanol. Cells were then washed three times with PBS and solubilized with a 1:1 ratio of PBS:absolute ethanol for 10 minutes with agitation. A_{600} was measured using a SpectraCount ELISA reader (Packard).

Results

Screening of the JCFN-RGD phage display library for αvβ3 binding resulted in selection of phage clones containing an RGDWXE in the FG loop of displayed FNfn10. In the FNfn10 structure the FG loop corresponds to residues 78-87, and the RGD sequence to residues 79-81 (residue numbering according to the Protein Data Bank entry 1TTG(14). The five residues immediately adjacent to the RGD sequence in the FG loop were modified. The JCFN-RGD (XRGDXXXX) library contained approximately 1.5 x 10^9 independent clones, sufficiently large to include all possible sequences. The library was amplified in 0.0, 0.1, or 1.0 mM IPTG to vary FNfn10 copy number and screened for binding to αvβ3 integrin. After 3 rounds of biopanning, 100-1000 fold increased recovery of phage was observed compared to the initial library (data not shown). Of 20 phage clones sequenced, 8 contained an RGDWXE consensus sequence (Figure 1a). Phage inputs of 10^9, 10^8, or 10^7 pfu were then allowed to bind to αvβ3 or BSA coated wells and bound phages were recovered with 0.2M glycine, pH2.2. Using phage clones amplified in 1 mM IPTG (high FNfn10 copy number), phage clones containing the RGDWXE consensus were recovered at levels of 0.01-1% input from αvβ3 coated wells; the clones were recovered at approximately 1000-fold lower levels from BSA coated wells and integrin-binding was found to be calcium-dependent (not shown). In contrast, clones which lacked the RGDWXE motif were recovered at equivalent levels from both αvβ3 coated and BSA coated wells (data not shown).

The RGDWXE-containing clones were amplified in the absence of IPTG so as to decrease the expressed copy number of FNfn10 protein on the phage surface, and the panel of clones were then directly compared in a phage binding assay, for their ability to bind to αvβ3 (Figure 1b). The clones recovered at the highest level were 2JCAV1, 2JCAV2, 3JCAV3, 3JCNI1, and

3JCLI4, all of which were recovered at a level of approximately 0.1% of input phage from αvβ3-coated wells and at 1000-fold lower levels from BSA-coated wells. Therefore, varying FNfn10 copy number on M13 by IPTG concentration did not have an effect on the relative binding efficiency of the clones isolated, suggesting that the binding interaction between the αvβ3 integrin and the phage-displayed FNfn10 derivatives might be of high affinity.

The modified FNfn10 proteins expressed by this panel of phage clones were expressed as an isolated protein in *E.coli*, purified and biotinylated. 3JCLI4 (the second-highest recovered clone in phage binding assays), was selected for further study because the purified FNfn10 protein derived from clone 2JCAV1 (the highest recovered clone) proved to be rather insoluble and prone to precipitation. SDS polyacrylamide gel electrophoresis and Coomassie blue staining revealed the presence of a single protein of the expected size (~15 kDa) within the purified preparation of biotinylated FNfn10-3JCLI4 protein. Western blot analysis using a monoclonal antibody specific for the hexahistidine epitope tag on the purified FNfn10 protein confirmed the identity of this protein (not shown), and further analysis revealed that (as expected) the binding of both the phage clone 3JCLI4 and of the corresponding purified protein (FNfn10-3JCLI4) to αvβ3 integrin was Ca^{++} dependent and could be eliminated in the presence of EDTA (data not shown).

FNfn10-3JCLI4 displays high affinity and specificity for plate-immobilized αvβ3. Biotinylated FNfn10-3JCLI4 and biotinylated wild-type FNfn10 (FNfn10-WT) were allowed to bind to αvβ3, α1β1, or BSA coated wells in approximate half-log increments from 0.0001 to 0.1 μg/ml, corresponding to molar concentrations of 8 pM to 8 nM. Bound biotinylated protein was detected by ELISA (Figure 2a). Assuming a high ratio of free to bound FNfn10-3JCLI4, the equation $y=m1*x / (m2 + x)$ was fit to the data ($R>0.99$) to determine the half-maximal binding affinity of

0.01 μg/ml or 800 pM. At each concentration examined, the binding of biotinylated FNfn10-3JCLI4 to αvβ3 was higher than that for FNfn10-WT, and 3JCLI4 was not found to bind detectably to the α1β1 integrin (i.e., binding to α1β1 was identical to binding to BSA).

We next examined the kinetics of protein association and dissociation. After allowing biotinylated FNfn10-3JCLI4 to bind to αvβ3-coated wells, 100 fold excess unlabelled FNfn10-3JCLI4 was added to some wells and the plates were incubated for a further 24 hours; control wells were incubated in the absence of the unlabelled competitor. At the end of this time, the amount of plate-bound biotinylated FNfn10-3JCLI4 was assessed by ELISA. No difference was detected between wells that were incubated for 24 hours in the presence or absence of excess unlabelled competitor protein. This suggests that the rate of dissociation of FNfn10-3JCLI4 from αvβ3 is very slow (>24 hours; Figure 2b).

To examine the rate of association between αvβ3 and biotinylated 3JCLI4, biotinylated FNfn10-3JCLI4, at concentrations of 0.01, 0.02, or 0.05 μg/ml, was allowed to bind to αvβ3-coated wells for periods of time ranging from one minute to two hours. For all three concentrations, levels of bound FNfn10-3JCLI4 were detectable within one minute and reached saturation levels within two hours (Figure 2c). Therefore, in light of the prolonged dissociation rate revealed in Figure 2b, we can infer that FNfn10-3JLCI4 possesses a relatively rapid on-rate for binding to αvβ3.

To test the specificity of the interaction between FNfn10-3JCLI4 and αvβ3 integrin, biotinylated FNfn10-3JCLI4 or FNfn10-WT were added to wells coated with 5 g/ml concentrations of different purified integrins (Figure 2d). In this assay, the detection of plate-bound FNfn10-3JCLI4 was significant only for those wells which had been coated with αvβ3, and not for wells coated with other integrins, including αvβ5, α5β1, α1β1 and αIIbβ3.

The disintegrin echistatin and FNfn10-3JCLI4, but not GRGDSPK peptide or FNfn10-WT, effectively compete with biotinylated FNfn10-3JCLI4 for αvβ3 binding. In order to examine the relative affinity of FNfn10-3JCLI4, as compared to other αvβ3 binding molecules, a competition ELISA analysis was performed. In these experiments, biotinylated FNfn10-3JCLI4 at 800 pM was mixed with molar ratios of competitor proteins ranging from 0.01 to 100, and allowed to bind to αvβ3 coated wells (Figures 3a and 3b). Binding of biotinylated FNfn10-3JCLI4 to αvβ3 was unaffected by the presence of the linear integrin-binding peptide (GRGDSPK) or FNfn10-WT, even when these molecules were added at 100-fold molar excess (80 nM), relative to FNfn10-3JCLI4. In contrast, unlabelled FNfn10-3JCLI4 and echistatin inhibited binding of biotinylated FNfn10-3JCLI4 at roughly equimolar concentrations (IC_{50} values were 750 and 930 pM, respectively, as calculated by logarithmic curve-fit). These findings strongly suggest that FNfn10-3JCLI4 and echistatin have nearly equivalent affinities for αvβ3 and bind to the same site; echistatin has a published Kd of 330 pM(20).

FNfn10-3JCLI4 is an effective and specific staining reagent, when used in flow cytometric analysis. Biotinylated FNfn10-3JCLI4 (1 μg/ml) was added to wild-type K562 cells and to K562 cells that had been stably transfected with αvβ3 integrin (generous gift of Dr. S. Blystone)(17). Surface bound FNfn10-3JCLI4 was then detected using streptavidin-APC and stained cells were enumerated by flow cytometry. The mean fluorescence intensity of staining on the αvβ3-positive cells was approximately three logs higher than on the non-transfected control cells (Figure 4a), and concentrations of biotinylated FNfn10-3JCLI4 as low as 8ng/ml were found to be capable of effectively and specifically staining K562-αvβ3 cells (data not shown).

Having optimized our staining analysis using the K562-αvβ3 cells, we proceeded to a more complete analysis of the specificity of cell surface binding of biotinylated FNfn10-3JCLI4, using

a panel of K562 cell sublines, each of which had been stably transfected with various integrin heterodimers (αvβ3, αvβ5, αIIbβ3, α4β1, and α4β7; gift of S. Blystone)(17). These experiments revealed that FNfn10-3JCLI4 showed a strong selective preference for αvβ3, although it did also react very weakly with αIIbβ3 positive cells. In contrast, FNfn10-WT showed a slight preference for cells expressing αvβ3, but stained all of the K562 lines with low intensity (Figure 4b).

One of the unique properties of the FNfn10 scaffold is its high level of physical stability. We therefore examined the effect of prolonged incubation at elevated temperatures on the binding activity of FNfn10-3JCLI4, as compared to the αvβ3s-specific monoclonal antibody, LM609. In these studies, FNfn10-3JCLI4 and LM609 were incubated for 24 hours at 4, 20, 37, 50, 65, or 75 degrees celsius, prior to addition to K562-αvβ3 cells and performance of flow cytometry. As can be observed in Figure 4c, incubation of biotinylated FNfn10-3JCLI4 at 75°C for 24 hours had only a modest effect on its cell binding activity; in contrast, the same conditions resulted in the complete elimination of LM609's ability to interact with αvβ3.

FNfn10-3JCLI4 inhibits αvβ3-dependent cell adhesion but not αIIbβ3-dependent cell adhesion. K562-αvβ3 cells, but not K562 cells, adhere strongly to vitronectin (but not BSA). This property was therefore used as the basis for a quantitative assessment of the ability of FNfn10-3JLCI4 to disrupt αvβ3 dependent cell adhesion. To do this, K562-αvβ3 cells were allowed to adhere to vitronectin-coated wells, and the number of bound cells per well was then quantified by crystal violet staining and cell solubilization.

LM609, a commonly used αvβ3 function-blocking antibody, and FNfn10-3JCLI4 were both found to inhibit adhesion of K562-αvβ3 cells to vitronectin, while FNfn10-WT showed little effect in this assay (Figure 5a). A more careful analysis, using a wider range of protein concentrations, revealed that FNfn10-3JCLI4 inhibits αvβ3-dependent adhesion to vitronectin at approximately

the same concentrations as the well-characterized, αvβ3-binding distintegrin, echistatin (Figure 5b). Based on an exponential curve-fit, echistatin was found to inhibit αvβ3-mediated cell adhesion to vitronectin with an IC_{50} of 5.9 nM, while FNfn10-3JCLI4 inhibited αvβ3-dependent adhesion to vitronectin with an IC_{50} of 8.6 nM ($R>0.99$ in both cases). Finally, we also examined the effect of FNfn10-3JCLI4 on αIIbβ3-dependent cell adhesion, because of the weak binding activity of FNfn10-3JCLI4 for K562-αIIbβ3 cells that was revealed in our flow cytometric analyses. These experiments revealed that FNfn10-3JCLI4 had no measurable effect on αIIbβ3-mediated cell adhesion to fibronectin, while echistatin inhibited αIIbβ3-dependent adhesion with an IC_{50} of 10.2 nM ($R>0.99$; Figure 5c).

Discussion

In this work, we have applied phage display technology and random mutagenesis techniques to carry out affinity maturation of FNfn10 binding to αvβ3 integrin. Using one phage clone that was derived from a biopanning screen against immobilized αvβ3, 3JCLI4, we found the following: (i) modified FNfn10 phage clones, including 3JCLI4, which bind to αvβ3 integrin all contain a RGDWXE consensus and exhibit calcium-dependent binding; (ii) purified FNfn10-3JCLI4 protein binds to immobilized human αvβ3 integrin with much higher affinity than FNfn10-WT and exhibits only background binding to other purified integrins; (iii) binding of FNfn10-3JLCI4 to αvβ3 could be successfully competed using approximately equimolar concentrations of unlabelled FNfn10-3JCLI4 or echistatin, but not by either FNfn10-WT or GRGDSPK peptide (even when added at 100-fold molar excess); (iv) FNfn10-3JCLI4 is a sensitive and specific reagent for the detection of human αvβ3 integrin in flow cytometry applications; (v) the binding of FNfn10-3JCLI4 to αvβ3 integrin is maintained even after prolonged exposure of FNfn10-3JCLI4 to high temperature, and (vi) FNfn10-3JCLI4 inhibits cell adhesion via αvβ3 but not αIIbβ3 integrin, at nanomolar concentrations.

The RGDW consensus sequence that was detected in our αvβ3-selected FNfn10-display phage clones has previously been reported to be preferentially selective for αIIbβ3 integrin over αvβ3, at least in the context of disintegrins such as eristostatin(21). RGDW peptides are also used to induce integrin activation and in competition assays with putative αIIbβ3 integrin ligands(22). Because FNfn10-3JCLI4 exhibits no binding to plate-immobilized αIIbβ3 and very low cross-reactivity with αIIbβ3–positive cells in flow cytometric analyis, the tertiary structure of FNfn10 may play a role in the increased specificity of this RGDW-containing molecule for αvβ3

integrin. Alternatively, the glutamic acid residue within the extended consensus motif (RGDWXE) may be significant. The disintegrins eristostatin and EC6B each contain an aspartic acid residue at the corresponding location (RGDWND)(21,23,24), and though they both bind αvβ3, they interact preferentially with αIIbβ3. It will be interesting to characterize the three-dimensional structure and conformational dynamics of the FNfn10 mutants obtained in this study.

Like the disintegrins, FNfn10-3JCLI4 is a small monomeric molecule with high integrin-binding affinity. In our studies, echistatin and FNfn10-3JCLI4 were inferred to have similar binding affinities for αvβ3 because they were both able to compete with biotinylated FNfn10-3JCLI4 for binding to immobilized αvβ3, at roughly equimolar concentrations. Consistent with this, echistatin has a reported K_d of 330 pM for αvβ3(20)while the value calculated here for the half-maximal binding affinity of FNfn10-3JCLI4 for αvβ3 was 800 pM.

Biochemical analyses revealed a rapid rate of association rate between FNfn10-3JCLI4 and αvβ3 (minutes), and a very slow rate of dissociation (> 24 hours). These properties, combined with its high affinity for αvβ3, suggested to us that FNfn10-3JCLI4 might be suitable for use in flow cytometric applications. Experimental analysis revealed this was indeed the case, and these studies also showed that FNfn10-3JCLI4 performed with similar specificity to an αvβ3-specific monoclonal antibody in flow cytometric staining using K562-αvβ3 cells. It should be noted that FNfn10-3JCLI4 is monomeric, unlike monoclonal antibodies (divalent) or other cell-staining reagents used in flow cytometry (MHC:peptide tetramers). Furthermore, FNfn10-3JCLI4 is considerably more heat stable than a typical monoclonal antibody, as revealed by its ability to continue to bind αvβ3, even after 24 hr. incubation at 75°C.

A problem encountered in many molecules that target αvβ3 integrins is their cross-reactivity with other integrin subtypes, particularly other αv or β3 integrins. FNfn10-3JCLI4 was found to

bind only to immobilized purified αvβ3, and not to other integrins tested. The failure of FNfn10-WT and FNfn10-3JCLI4 to interact with α5β1 is consistent with previous reports that the ninth and eighth fibronectin type III domains are also required for FNfn10 binding to α5β1(25). In addition, the inability of FNfn10-3JCLI4 to bind to αvβ5 and αIIbβ3 suggests that its integrin-binding activity is likely dependent on a structural interaction with the αvβ3 complex and not with either subunit alone. The narrow integrin-binding specificity of FNfn10-3JCLI4 can be contrasted with the more broad integrin-binding profile that is exhibited by disintegrins such as eristostatin and kistrin, which bind to both αIIbβ3 and αvβ3(21). Similarly, the penton base protein from adenovirus type 5 binds to both αvβ3 and αvβ5, and even an engineered Fab construct (WOW-1) has been shown to exhibit cross-reactivity with αvβ5 integrin(26).

FNfn10-3JCLI4 did exhibit a weak interaction with αIIbβ3 in our flow cytometry assay, but we do not anticipate that this will be a major detriment to the more widespread use of this molecule in cell staining applications because (i) no interaction with αIIbβ3 was detected in biochemical binding studies (see above), (ii) binding to αIIbβ3 positive cells was almost 10-fold weaker than binding to αvβ3-positive cells in the flow cytometric analysis, and (iii) αIIbβ3 expression is limited to platelets. The ability of FNfn10-3JCLI4 to block αvβ3–dependent cell adhesion but not αIIbβ3-mediated cell binding further supports its specificity for αvβ3.

The structure of FNfn10-3JCLI4 is advantageous for multiple applications; FNfn10-3JCLI4 lacks disulfide bonds rendering it resistant against reducing agents, is very stable even at high temperature, and can be produced in a bacterial expression system with yields of up to 50-100 mg per liter of culture. As an endogenous human protein, wild-type fibronectin does not elicit an immune response and it is anticipated that FNfn10-3JCLI4 is likely to be similarly non-immunogenic. Furthermore, because FNfn10-3JCLI4 is a monomer that lacks disulfide bonds, it has several advantages over a monoclonal antibody with similar affinity. As a small, single-chain

molecule, FNfn10-3JCLI4 could potentially be incorporated into gene delivery vectors (e.g. viral vectors, liposomes) for cell or tissue-specific gene expression. Indeed, the incorporation of low-affinity RGD peptides into various viral vectors, and the addition of bispecific antibody complexes to viral surface components, have already been shown to result in desirable improvements in vector specificity and transduction efficiency(27-30). αvβ3-positive cells that may be of particular interest for targeted vector systems include dendritic cells (for vaccine applications) as well as osteoclasts and angiogenic vessels. Finally, FNfn10-3JCLI4 may have utility as a cancer imaging agent, because of its ability to recognize αvβ3 on tumor vasculature. Further studies will be required to explore these various applications.

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Figure Legends

Figure 1. Phage display screening of JCFN-RGD library. M13 Phage displaying a library of FNfn10 clones with partially randomized FG loops (XRGDXXXX) were amplified using 0.0, 0.1, or 1 mM IPTG to vary FNfn10 copy number on the phage. Amplified phage populations were subjected to three rounds of biopanning against wells coated with αvβ3 integrin (5 μg/ml). (a) A schematic drawing of FNfn10 (14) showing the RGD sequence and the positions of residues in the FG loop that were diversified in the JCFNRGD library. The figure was made with the program MOLSCRIPT(31). (b) Five clones each were sequenced from the second round eluate of the library amplified with 1mM IPTG (2JCAV1-2JCAV5), from the third round eluate of the library amplified with 1 mM IPTG (3JCAV1-5), from the third round eluate of the library amplified with low IPTG (3JCLI1-5), and from the third round eluate of the library amplified with no IPTG (3JCNI1-5). (c) Phage binding assay of the phage clones containing the WXE consensus and unmodified FNfn10 (pVIII-FNfn10), all amplified without IPTG. Phage inputs of 10^9, 10^8, or 10^7 pfu were added to wells coated with 5 μg/ml αvβ3 (solid bars) or 0.5mg/ml BSA (patterned bars). After one hour, non-binding phage were removed by extensive rinsing and bound phage were eluted with 0.2M glycine, pH 2.2. Eluates from each well were titered. Results shown are representative of three different experiments that yielded similar results.

Figure 2. Assessment of FNfn10-3JCLI4 binding affinity to αvβ3 integrin by ELISA. (a) To determine the half-maximal binding concentration of biotinylated FNfn10-3JCLI4 versus biotinylated FNfn10-WT, wells of a 96-well plate were coated αvβ3 (5 μg/ml), α1β1 (5 μg/ml), or BSA (0.5 mg/ml) and blocked with BSA. Biotinylated FNfn10-3JCLI4 and FNfn10-WT were then

added to the wells and allowed to bind for two hours. Bound protein was detected by streptavidin-horseradish peroxidase (2 μg/ml) and ABTS substrate. O.D. $_{400}$ measurements were recorded for biotinylated FNfn10-3JCLI4 binding to αvβ3 (◊), to α1β1 (◆), or to BSA (×) and biotinlated FNfn10-WT binding to αvβ3 (□). Error bars reflect the standard deviation between triplicate wells. (b) To examine protein dissociation over a prolonged (24 hour) time period, a 96-well plate was coated with 5 μg/ml αvβ3 integrin and then incubated with 0.02 μg/ml biotinylated FNfn10-3JCLI4 for two hours; as a control, 10 mM EDTA was added to certain wells, in order to calculate the background level of FNfn10-3JCLI4 binding. After rinsing, 2 μg/ml unlabelled FNfn10-3JCLI4 was added to the preformed protein complexes present in the wells 24, 14, 4, 2 and 1 hours prior to detection of remaining plate-bound biotinylated FNfn10-3JCLI4 using streptavidin-horseradish peroxidase and ABTS. Baseline binding activity (100%) was calculated as the amount of biotinylated FNfn10-3JCLI4 which remained bound to the plate following incubation of the well for 24 hours, in the presence of buffer alone (i.e., in the absence of any competitor protein), and the O.D. $_{400}$ of FNfn10-3JCLI4 bound in the presence of 10 mM EDTA was subtracted as background. All incubations with competitor (background-subtracted) are expressed as a percentage of the O.D. $_{400}$ of baseline binding (background-subtracted). Error bars reflect the standard deviation between triplicate wells. (c) To examine rate of association of αvβ3 and biotinylated FNfn10-3JCLI4, a 96-well plate coated with 5 μg/ml αvβ3 integrin was blocked with BSA. 0.01 (▲), 0.02 (■), or 0.05 (◆) μg/ml of biotinylated FNfn10-3JCLI4 was then added to the coated wells, at 240, 30, 10, 3, and 1 minute prior to washing and detection. After detection with streptavidin-horseradish peroxidase/ABTS, the O.D. $_{400}$ of the plate was recorded. Error bars reflect the standard deviation between triplicate wells. (d) A 96-well plate was coated with αvβ3, αvβ5, αIIbβ3, α1β1, and α5β1 purified integrins, all at 5 μg/ml. Biotinylated FNfn10-3JCLI4 (dark bars) or FNfn10-WT (light bars) was then added at a concentration of 0.01 μg/ml,

and allowed to incubate for 2 hours, followed by washing and detection. Error bars reflect the standard deviation between triplicate wells. (a-d): the experiments shown are all representative of three different experiments that yielded similar results.

Figure 3. Competition ELISAs to determine relative affinities of FNfn10-3JCLI4, echistatin, FNfn10-WT, and GRGDSPK peptide. A BSA-blocked 96-well plate was coated with αvβ3 at 5 μg/ml, and biotinylated FNfn10-3JCLI4 was then added in the presence or absence of a 0.01, 0.1, 1, 10, or 100-fold molar excess of unlabelled competitor protein, and incubated for two hours. After washing and detection, the $O.D._{400}$ of the plate was recorded. Error bars reflect the standard deviation between triplicate wells, and the results shown are representative of three different experiments that yielded similar results. A background subtraction was performed on all data points, by subtracting the measured $O.D._{400}$ value that corresponded to binding of biotinyated FNfn10-3JCLI4 in the presence of 10 mM EDTA. All values in the figure are expressed as a percentage of the $O.D._{400}$ of biotinylated FNfn10-3JCLI4 binding in the absence of competitor. (a) Binding competition with unlabelled FNfn10-3JCLI4 (◆) or unlabelled FNfn10-WT (■). (b) Binding competition with unlabelled FNfn10-3JCLI4 (▲), echistatin (■), and GRGDSPK peptide (◆).

Figure 4. Fluorescence activated cell sorting (FACS) analysis of FNfn10-3JCLI4 binding to human cells. Biotinylated FNfn10-3JCLI4 or FNfn10-WT were added to 10^5 cells at a concentration of 1 μg/ml and incubated for 30 minutes. Bound proteins were then detected with streptavidin-APC, and the mean fluorescence of ten thousand cells per sample was recorded. All experiments were performed three times, with similar results; error bars (b, c) represent the standard deviation of three samples of 10^5 cells stained separately. (a) Histogram plot of flow

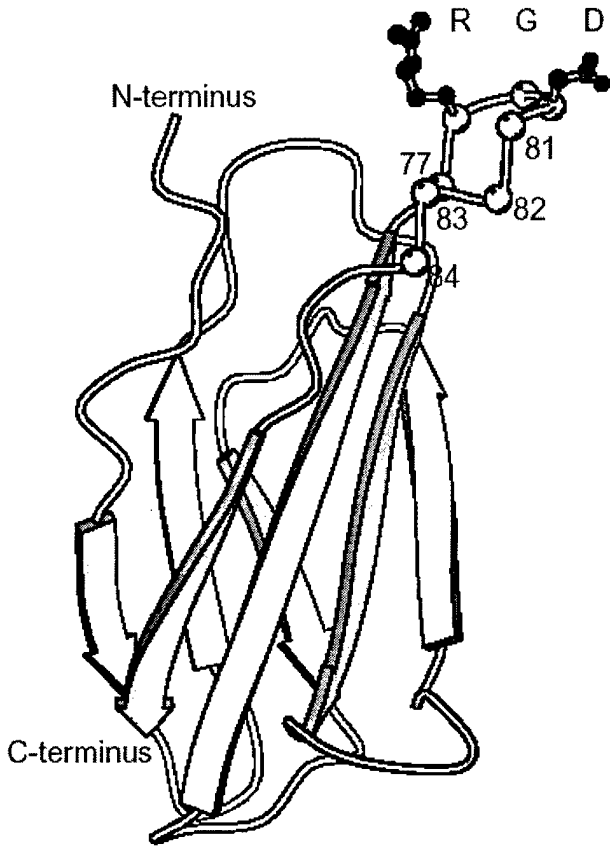
cytometric staining analysis of K562 and K562-αvβ3 cells using FNfn10-3JCLI4. (b) Mean APC fluorescence for integrin-transfected K562 cells stained with biotinylated FNfn10-3JCLI4 (dark bars) or FNfn10-WT (light bars). (c) To determine the effect of high temperature on FNfn10-3JCLI4 stability, 1 μg /ml 3JCLI4 (◆) or 5 μg /ml LM609 (■) were incubated for 24 hours in FACS buffer at 4, 20, 37, 50, 65, or 75°C. 10^5 K562-αvβ3 cells were pelleted for each sample and resuspended in the appropriately treated FNfn10-3JCLI4 or LM609, followed by detection as above.

Figure 5. Inhibition of integrin-dependent cell adhesion. Vitronectin (a) or fibronectin (b, c) coated strips (as well as uncoated control wells) were blocked with BSA and incubated with 10^5 K562 cells, K562-αvβ3 cells, or K562-αIIbβ3 cells for two hours in the presence or absence of the indicated inhibitors. Non-adherent cells were removed by washing, while adherent cells were stained with crystal violet, solubilized, and quantitated by measuring the $O.D._{570}$. The background level of crystal violet in control wells was calculated on the basis of measured $O.D._{570}$ for BSA-coated wells (no integrin). Error bars represent the standard deviation of triplicate wells, and all experiments were performed three times, with similar results. (a) Adhesion of binding of K562-αvβ3 cells to vitronectin. Inhibitors added include FNfn10-3JCLI4, FNfn10-WT, and LM609 antibody, at the stated final concentrations. The lane marked "no αvβ3" corresponds to control K562 cells (no αvβ3), while "no VN" denotes cell adherence (K562-αvβ3 cells) measured on control wells (no vitronectin); the lane marked "NT" corresponds to K562-αvβ3 cells which were bound to vitronectin in the absence of any competitor. (b) Adhesion of K562-αvβ3 cells to vitronectin. Echistatin (■) and FNfn10-3JCLI4 (◆) were added at final concentrations of 400, 40, and 4 nM, prior to addition of the cells and initiation of the binding assay. Binding is expressed as a percentage of the $O.D._{570}$ recorded for K562-αvβ3 adhesion in

the absence of competitor, and a background subtraction was performed for all data points, which corresponded to the measured O.D. $_{570}$ for BSA-coated wells (no integrin). (c) Adhesion of K562-αIIbβ3 cells to fibronectin. Echistatin (■) and FNfn10-3JCLI4 (◆) were added at final concentrations of 400, 40, and 4 nM prior to addition of the cells and initiation of the binding assay. Binding is expressed as a percentage of the O.D. $_{570}$ recorded for K562-αIIbβ3 adhesion in the absence of competitor, and a background subtraction was performed for all data points, which corresponded to the measured O.D. $_{570}$ for BSA-coated wells (no integrin).

Fibronectin type III domain with enhanced binding to $\alpha v\beta 3$

Figure 1a:



Fibronectin type III domain with enhanced binding to $\alpha v\beta 3$

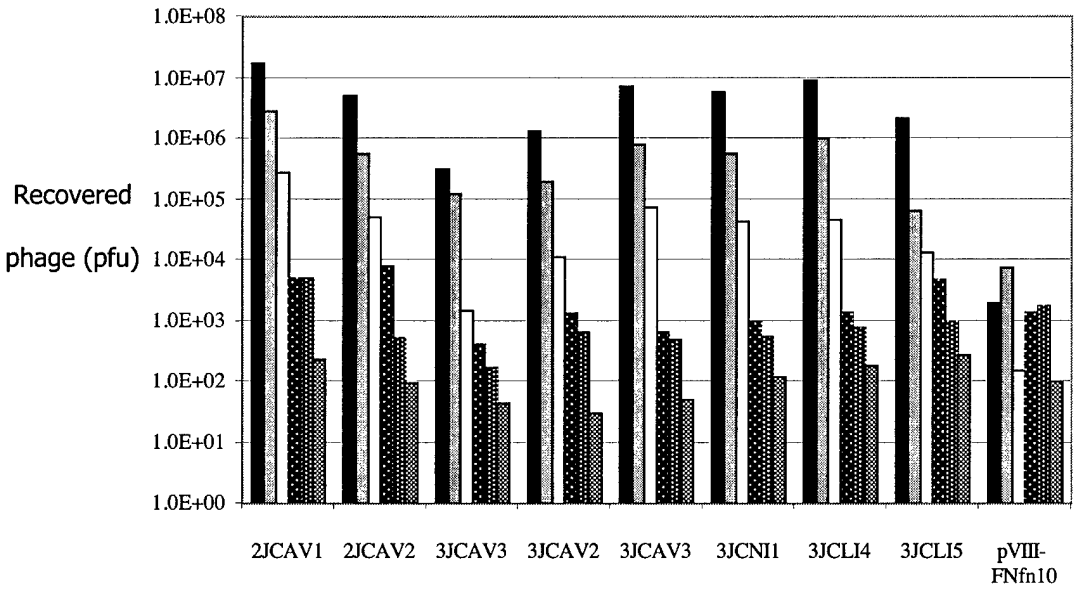
Figure 1b:

2JCAV1: LRGD**W**S**E**D	**3JCNI1**: PRGD**W**I**E**F
2JCAV2: VRGD**W**Y**E**Y	3JCNI2: GRGDSPAS
2JCAV3: VRGDCSSS	3JCNI3: GRGDDDRL
2JCAV4: GRGDLCDF	3JCNI4: GRGDYVLG
2JCAV5: GRGDSPAS	3JCNI5: GRGDFSFL
3JCAV1: GRGD**W**T**E**H	3JCLI1: SRGDVVPP
3JCAV2: ARGD**W**V**E**G	3JCLI2: TRGDPPPH
3JCAV3: PRGD**W**T**E**G	3JCLI3: SRGDVVPP
3JCAV4: GRGDAFSL	**3JCLI4**: GRGD**W**N**E**G
3JCAV5: FRGDSPLD	**3JCLI5**: FRGD**W**I**E**L

Wild-type FNfn10:

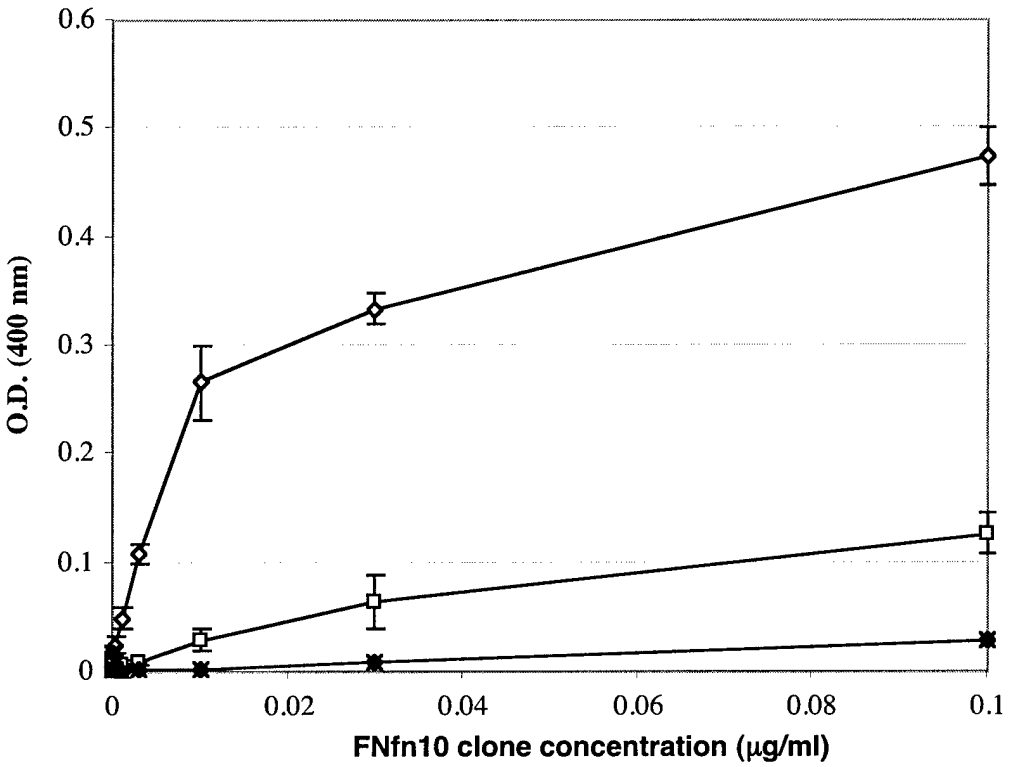
GRGDSPAS

Figure 1c:



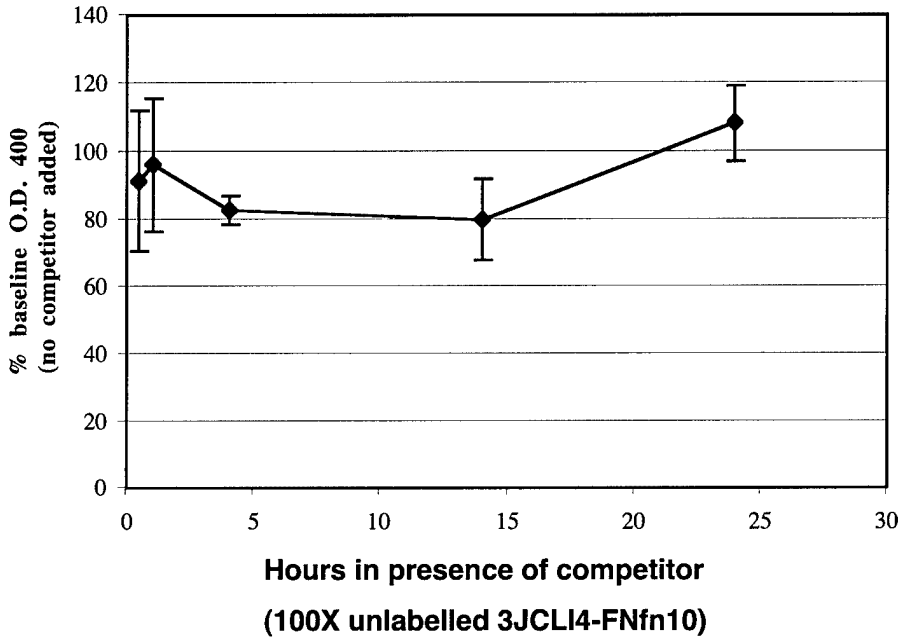
Fibronectin type III domain with enhanced binding to αvβ3

Figure 2a:



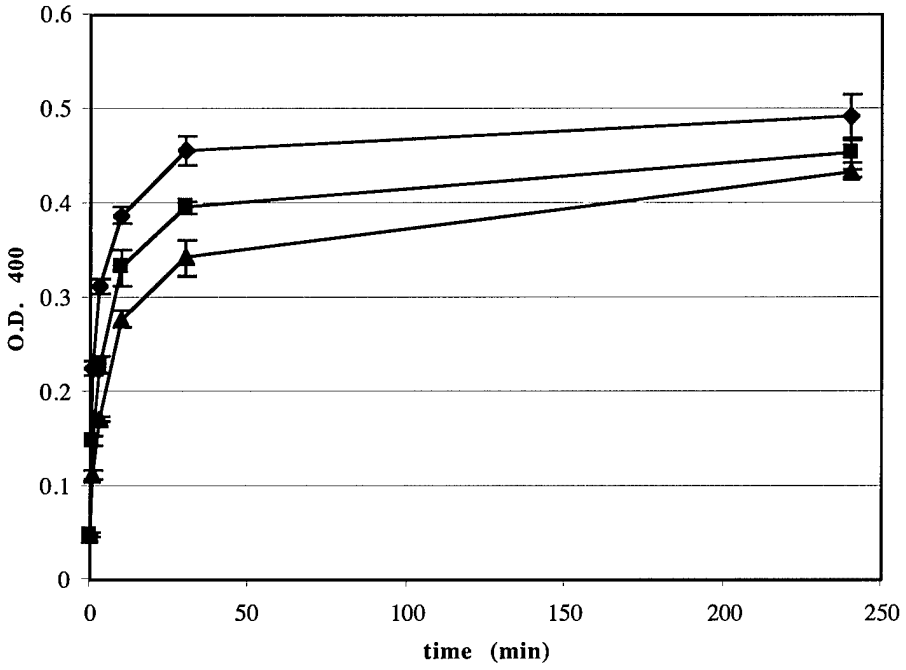
Fibronectin type III domain with enhanced binding to αvβ3

Figure 2b:



Fibronectin type III domain with enhanced binding to αvβ3

Figure 2c:



Fibronectin type III domain with enhanced binding to αvβ3

Figure 2d:

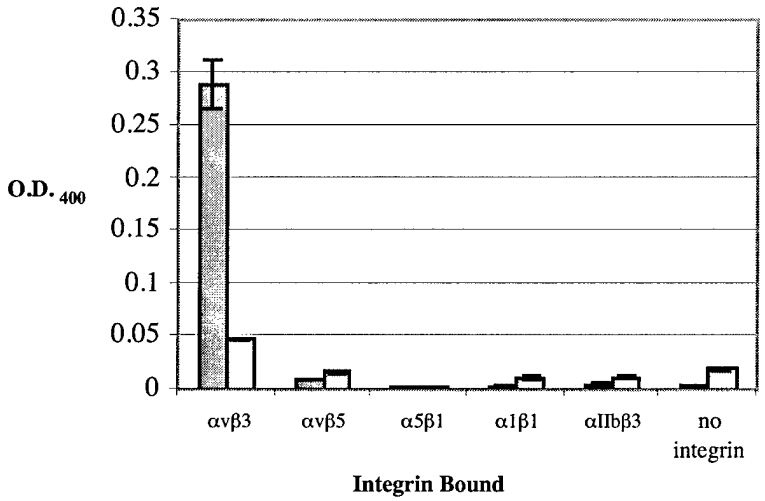


Figure 3a:

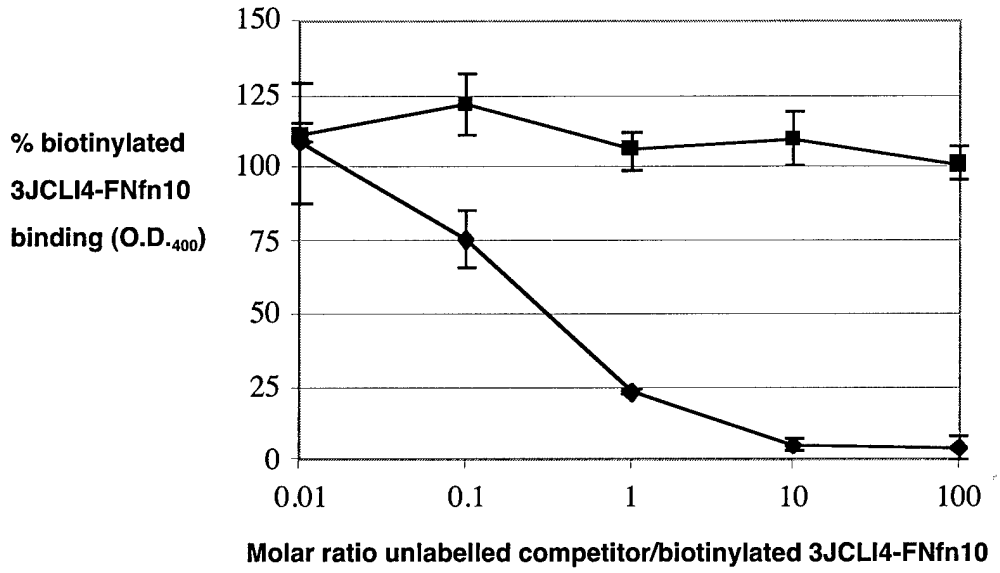
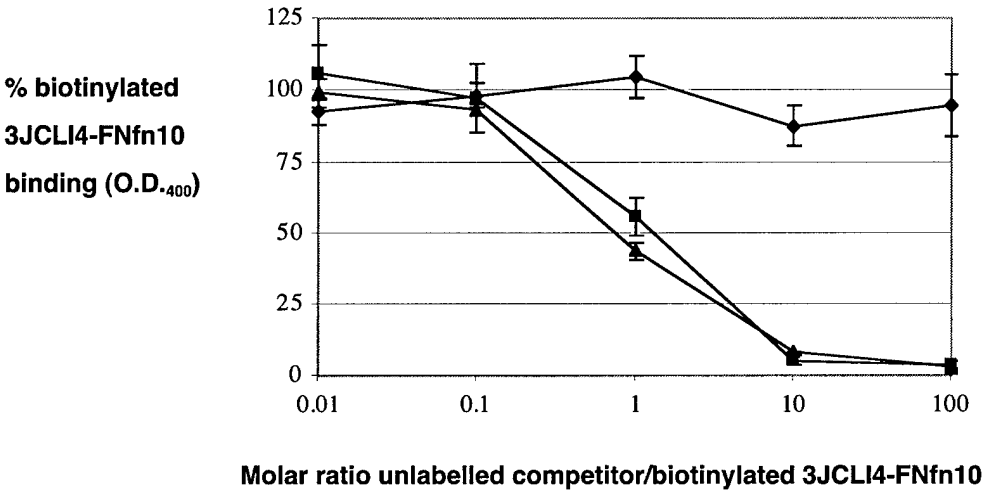
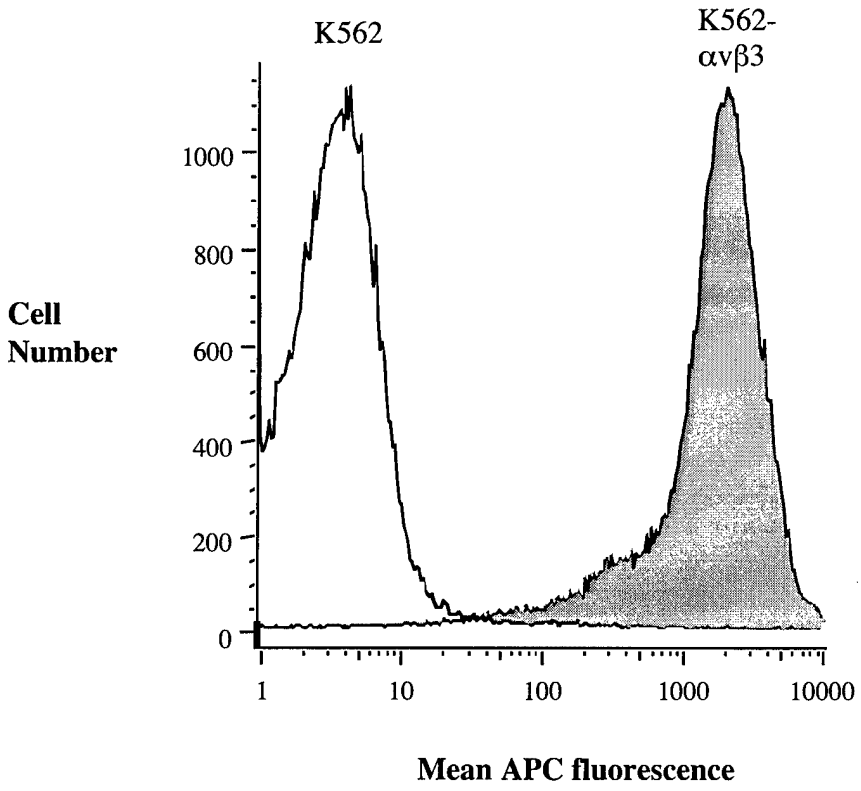


Figure 3b:



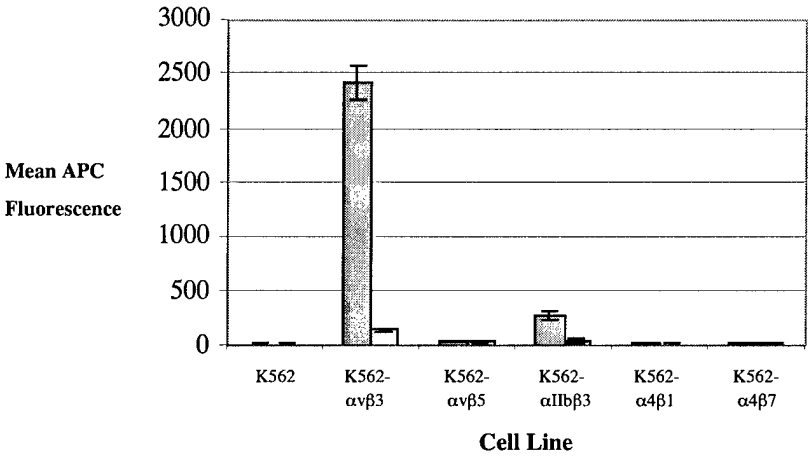
Fibronectin type III domain with enhanced binding to αvβ3

Figure 4a:



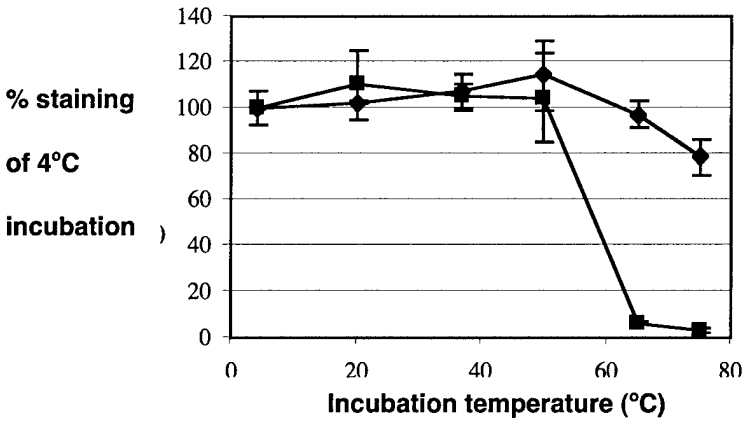
Fibronectin type III domain with enhanced binding to αvβ3

Figure 4b:



Fibronectin type III domain with enhanced binding to αvβ3

Figure 4c:



Fibronectin type III domain with enhanced binding to αvβ3

Figure 5a:

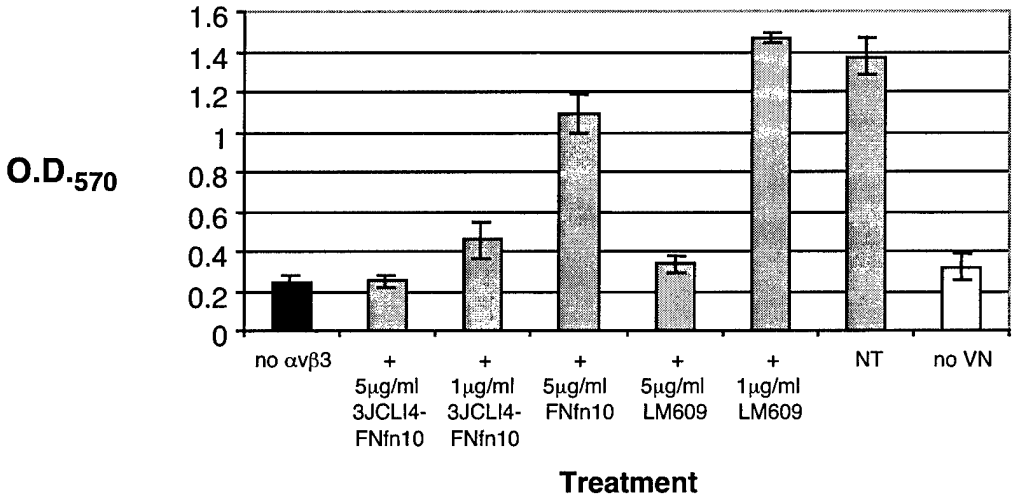


Figure 5b:

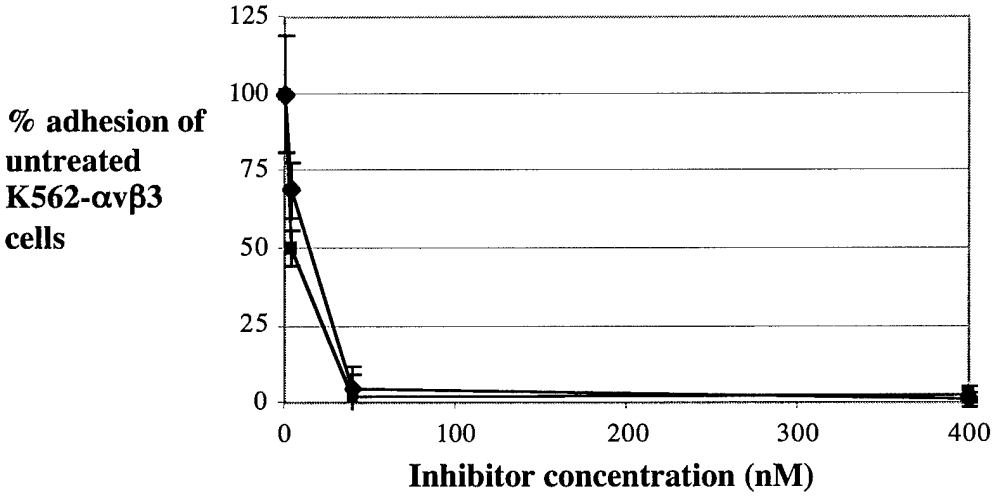
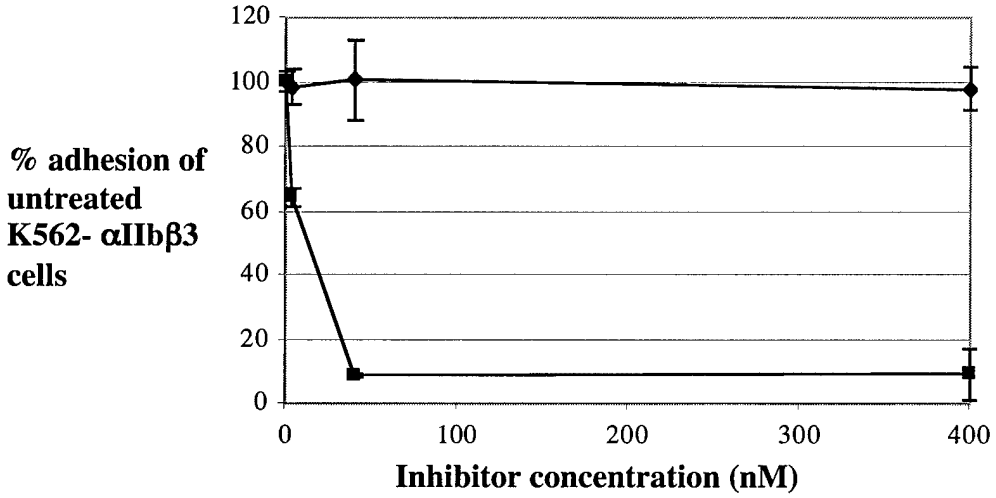

% adhesion of untreated K562-αvβ3 cells
125
100
75
50
25
0
0
100
200
300
400
Inhibitor concentration (nM)

Figure 5c:


% adhesion of untreated K562- αIIbβ3 cells
120
100
80
60
40
20
0
0
100
200
300
400
Inhibitor concentration (nM)